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Short communication

Preparative separation of isoquinoline alkaloids from *Stephania yunnanensis* by pH-zone-refining counter-current chromatography

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ABSTRACT

In this paper, five isoquinoline alkaloids were successfully separated from a crude extract of *Stephania yunnanensis* using pH-zone-refining counter-current chromatography in single-step. With a two-phase solvent system composed of methyl-*tert*-butyl ether (MtBE)–acetonitrile–water (2:2:3, v/v) where triethylamine (10 mM) was added to the upper organic phase as a retainer and hydrochloric acid (5 mM) to the aqueous mobile phase as an eluter. From 1.4 g crude extract, 68.7 mg isocorydine, 78.2 mg corydine, 583.4 mg tetrahydropalmatine, 36.3 mg N-methylasimilobine, and 47.3 mg anonaine were separated with purities over 90%. Their structures were identified by ^{1}H NMR, ^{13}C NMR, ESI-MS data.

1. Introduction

Stephania yunnanensis belongs to the family of Menispermaceac, and is rich in Yunnan province, China. The plants of this genus often have high alkaloid content, with strong activity and high medicinal value. According to previous research, *S. yunnanensis* contains several isoquinoline alkaloids, such as sinoacutine, L-tetrahydropalmatine, stephanine, stepharanine, and crebanine [1,2]. Pharmacological studies have demonstrated that these isoquinoline alkaloids have antitumor, antifibrotic, antimalarial, and anti-HIV activities [3–6]. In view of these beneficial effects, an efficient method for the preparative separation and purification of these alkaloids from natural sources is warranted. The preparative separation and purification of alkaloids from *S. yunnanensis* by conventional methods are tedious and usually requires multiple chromatography steps [1,7]. Counter-current chromatography (CCC) is a support-free liquid–liquid partition chromatography without use of solid support matrix [8]. Therefore, it eliminates the complications resulting from the solid support matrix, such as irreversible adsorptive sample loss and deactivation, tailing of solute peaks, and contamination. pH-zone-refining CCC was developed by Ito in 1990s [9–11]. The method enables separation of organic acids and bases into a succession of highly concentrated rectangular peaks with minimum overlap that elute according to their pK_a values and hydrophobicities. Compared to traditional CCC, it has many important advantages including an over 10-fold increase in sample loading capacity, high concentration of fractions, concentration of minor impurities, etc. In previous research, this method has been successfully applied to the analysis and separation of various natural and synthetic products, especially for alkaloids and organic acids [11–16]. So far, no report has been published on the use of CCC for the isolation and purification of isoquinoline alkaloids in *S. yunnanensis*. The purpose of this study, therefore, is to develop a CCC method for preparative isolation and purification of major alkaloids from the ethanol extract of *S. yunnanensis*.

2. Experiments

2.1. Reagents and materials

Methyl-*tert*-butyl ether (MtBE), acetonitrile, hydrochloric acid and triethylamine (TEA) used for CCC were of analytical grade and purchased from Huadong Chemicals, Hangzhou, China. Reverse osmosis Milli-Q water (18 MΩ) (Millipore, Bedford, MA, USA) was used for all solutions and dilutions. Acetonitrile used for HPLC analysis was of chromatographic grade and purchased from Merck, Darmstadt, Germany. The dried roots of *S. yunnanensis* were purchased from a local drug store.

2.2. Apparatus

The separations were performed on a semi-preparative apparatus (Ito scheme IV [17]) with one 140 mL coil and a counterweight. This instrument was manufactured by the Zhejiang University

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machine shop (Hangzhou, China). The multilayer coil was prepared by winding a 26.4 m × 2.6 mm i.d. PTFE tube. The β-value varied from 0.33 at the internal terminal to 0.60 at the external terminal ($\beta = r/R$ where r is the distance from the coil to the holder shaft, and R is the revolution radius or the distance between the holder axis and central axis of the centrifuge). The revolution speed of the apparatus can be regulated with a speed controller in the range of 0 and 1000 rpm and the sample injection was accomplished by an injection valve with a 10-mL sample loop. Furthermore, a Model 2W-2B constant-flow pump (Beijing Xingda Equipment, Beijing, China) was used to fill the CCC apparatus with the stationary phase and to elute the mobile phase. The effluent was continuously monitored by an HD-9704 UV spectrometer (Jingke Equipment, Shanghai, China) operating at 254 nm. Eluent was collected by a BSZ-100 fraction collector and a N2000 data analysis system (Institute of Automation Engineering, Zhejiang University, Hangzhou, China) was used to record the CCC chromatogram. A PHS-3B pH Meter (Shanghai Precision & Scientific instrument, Shanghai, China) was used for pH measurement.

2.3. Preparation of crude extract

The roots of *S. yunnanensis* were dried to constant mass at 55 °C in a vacuum oven and then pulverized. One kilogram of *S. yunnanensis* powder was extracted by 5 L of 95% ethanol for 2 h under reflux. The procedure was repeated for three times. The combined 15 L ethanol solution was concentrated to dryness under reduced pressure at 45 °C producing about 100 g of ethanol extract, of which 10 g ethanol extract was dissolved with 250 mL of 1% HCl. After filtration the residue was removed, and the pH of acidic aqueous solution was adjusted to 10 with NaOH solution. After filtration and air-drying, 2 g residue was obtained and was stored in a refrigerator (4 °C) for the subsequent pH-zone-refining CCC separation.

2.4. Preparation of the two-phase solvent systems and sample solution

The two-phase solvent system used for CCC separation was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated. TEA was added to the upper phase at 10 mM as a retainer with pH 2.38 and HCl was added to the lower phase at 5 mM as an eluter with pH 10.19.

The sample solution was prepared by dissolving 1.4 g crude sample in 10 mL of the upper phase (10 mM TEA) with pH 9.55.

2.5. Separation procedure

For each separation, the CCC column was first entirely filled with the upper organic phase. Then the crude sample which dissolved in upper phase was injected through the sample port and the aqueous mobile phase was pumped through the column at a flow rate of 1.5 mL/min in the head to tail direction (reversed mode), while the column was rotated at 600 rpm. The effluent was monitored continuously at 254 nm and automatically collected in test tube per 5 min using a BSZ-100 fraction collector. After the separation was completed, retention of the stationary phase was measured by collecting the column contents into a graduated cylinder by forcing them out of the column with pressurized nitrogen gas. Peak fractions were analyzed by HPLC.

2.6. HPLC analysis and identification of CCC peak fractions

HPLC analyses of the crude sample and CCC peak fractions were performed with a Hypersil reverse phase C18 column (250 mm × 4.6 mm i.d., 5 μm, Yilite). The mobile phase was acetonitrile (solvent A) and 0.1% TEA (solvent B) at the following

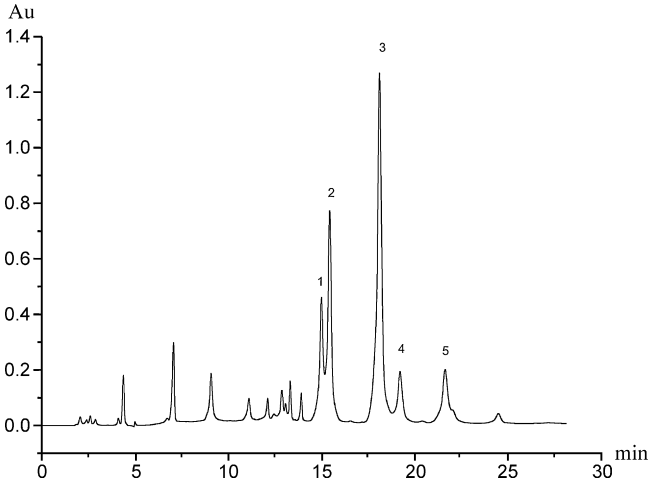

Fig. 1. Chromatogram of the crude extract from *Stephania yunnanensis* by HPLC. Column: Hypersil reverse phase C18 column (250 mm × 4.6 mm i.d., 5 μm, Yilite). The mobile phase was acetonitrile (solvent A) and 0.1% TEA (solvent B) at the following gradient: 0 min 5% A; 7 min 45% A; 25 min 55% A; detection: 280 nm; flow rate: 0.8 mL/min.

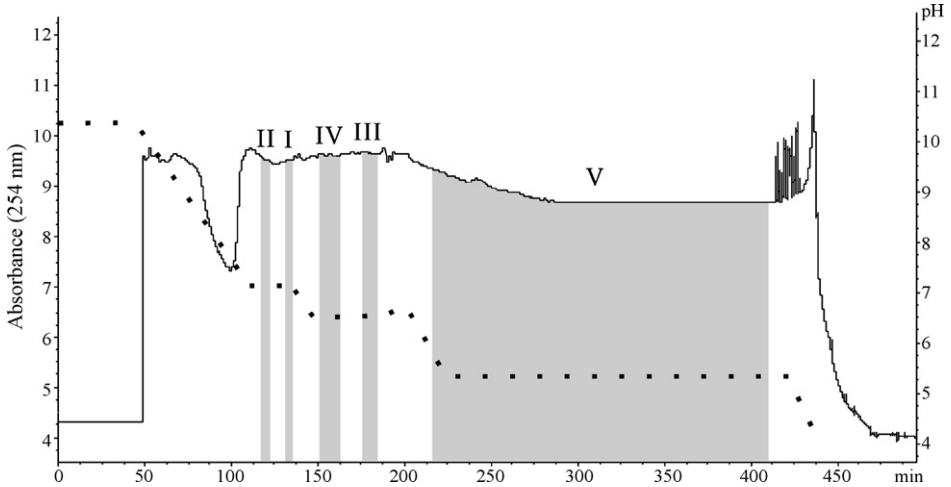

Fig. 2. Chromatogram of the crude extract from *Stephania yunnanensis* by pH-zone-refining CCC. Solvent system: MtBE–CH_3CN–water (2:2:3, v/v), 10 mM TEA in the upper organic stationary phase and 5 mM HCl in the lower aqueous phase; elution mode, head to tail; flow rate: 1.5 mL/min; detection: 254 nm; revolution speed: 600 rpm; retention of stationary phase 34%.

gradient: 0 min 5% A; 7 min 45% A; 25 min 55% A. The flow rate was 0.8 mL/min and was monitored at 280 nm.

Identification of the CCC peak fraction was performed by ESI-MS, ^{1}H NMR and ^{13}C NMR. ESI-MS analyses were performed using Bruker Esquire 3000 plus spectrometer with an electrospray ionization (ESI) interface in the positive mode. NMR experiments were carried out using a Bruker Avance DMX 500 NMR spectrometer with chloroform ($CDCl_3$) or dimethyl sulfoxide (DMSO) as solvent and TMS as internal standard.

3. Results and discussion

The composition of mobile phase influences the quality of HPLC separation. Different mobile phase composition, such as aqueous acetonitrile, aqueous methanol, aqueous methanol containing 0.1% triethylamine and aqueous acetonitrile containing 0.1% triethylamine were evaluated. Acetonitrile led to a better resolution of separation than methanol, and the peak shape was improved with additive TEA in aqueous phase. Hence aqueous acetonitrile con-

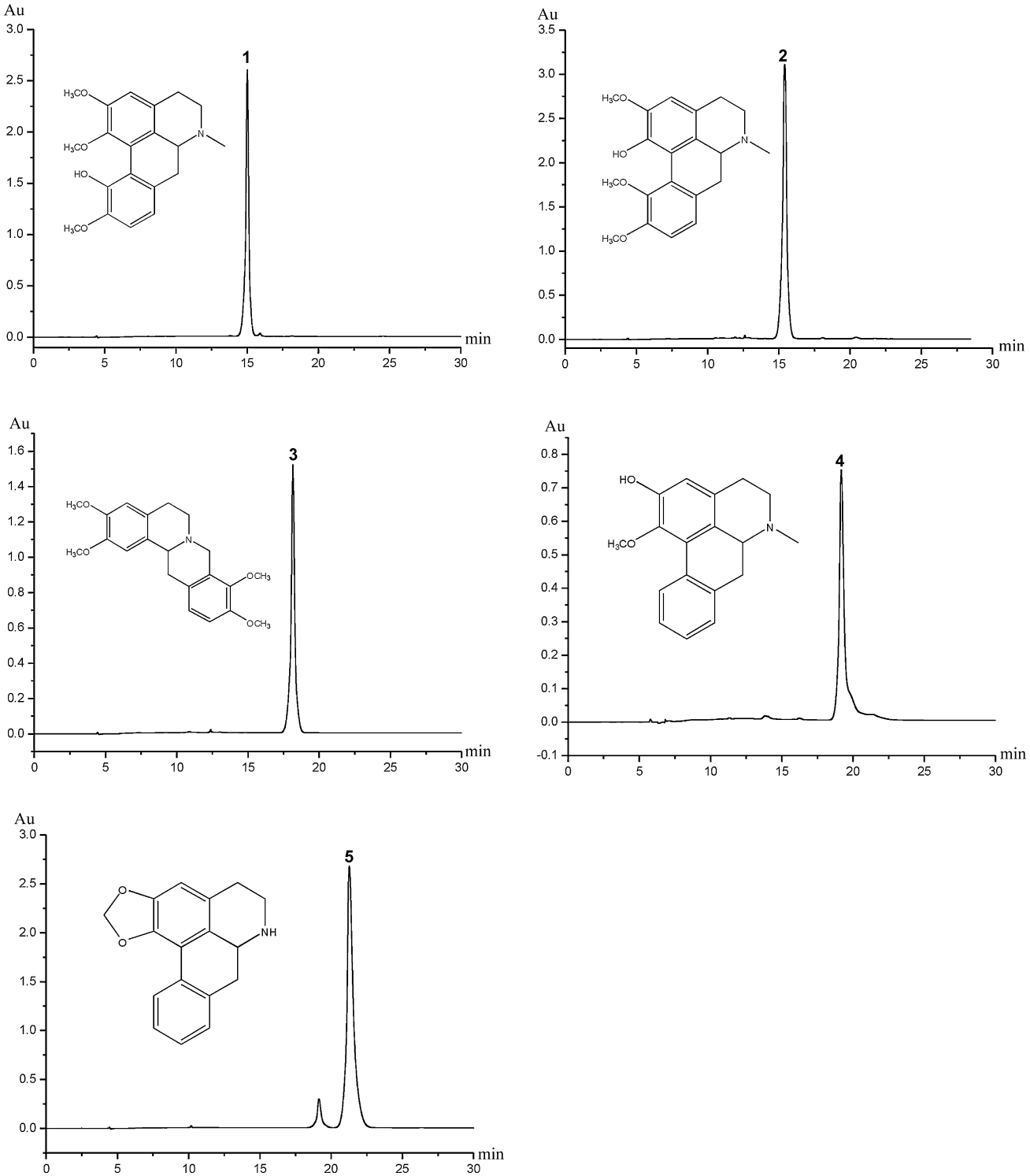

Fig. 3. HPLC analysis of each CCC fractions. Column: Hypersil reverse phase C18 column (250 mm × 4.6 mm i.d., 5 μm, Yilite). The mobile phase was acetonitrile (solvent A) and 0.1% TEA (solvent B) at the following gradient: 0 min 5% A; 7 min 45% A; 25 min 55% A; detection: 280 nm; flow rate: 0.8 mL/min. The structure of the purified compound was shown in the corresponding HPLC chromatogram.

taining 0.1% triethylamine was adopted in subsequent studies. The crude sample obtained from *S. yunnanensis* was first analyzed by HPLC, and the chromatogram is shown in Fig. 1.

For the present application with alkaloid components, a suitable two-phase solvent system was necessary which should provide ideal partition coefficient (K) values in both acidic ($K_{acid} \ll 1$) and basic ($K_{base} \gg 1$) conditions as well as good solubility of the sample in the solvent system [18]. We first evaluated a binary two-phase solvent system composed of MtBE–water (1:1, v/v) which has been used for many kinds of compounds [10,18,19]. Although this solvent system produced suitable K values ($K_{acid} = 0.0049$, $K_{base} = 23.27$), the maximum sample possible to separate in one experiment was 0.4 g due to poor solubility of the sample in this solvent system. The solubility of the sample was substantially improved by adding CH_3CN to the above solvent system, and the solvent system was optimized by selecting MtBE–CH_3CN–water (2:2:3, v/v). The crude sample was well dissolved in this solvent system and the K values were also suitable ($K_{acid} = 0.32$, $K_{base} = 26.38$).

Fig. 2 shows the preparative pH-zone-refining CCC separation of 1.4 g crude sample using the solvent system composed of MtBE–CH_3CN–water (2:2:3, v/v). The retention of stationary phase was 34%. Each CCC fraction was analyzed by HPLC and the chromatograms are shown in Fig. 3. As a result, 36.3 mg N-methylasimilobine ($pK_a = 7.88$), 47.3 mg anonaine ($pK_a = 8.29$), 78.2 mg corydine ($pK_a = 6.77$), 68.7 mg isocorydine ($pK_a = 6.78$) and 583.4 mg tetrahydropalmatine ($pK_a = 6.53$) were obtained from zone I to zone V, respectively, with over 90% purity in a single-step separation. The pK_a values of the purified compounds were obtained by ACD/Labs software V8.14 (pK_a). The pK_a values of solutes play an important role in pH-zone-refining CCC separation. The solutes elute according to their pK_a values and hydrophobicities. The elution order was slightly different from their pK_a value due to their different hydrophobicities. Compared with traditional CCC method, pH-zone-refining CCC extends the preparative capacity. In this work, 1.4 g crude sample was separated with a semi-preparative CCC instrument (the volume of column is 140 mL) using pH-zone-refining CCC, the throughput was about 73 mg/h. Compared with previous research [16], the sample used in this study was more complex. Two similar structures corydine and isocorydine were separated successfully which indicating this method was suitable for separation of isoquinoline alkaloids.

The structural identification of CCC fractions in Fig. 2 was carried out by positive ESI-MS, ^{1}H NMR, and ^{13}C NMR as follows:

Compound **1**: positive ESI-MS, m/z 342 (M+H). ^{1}H NMR (500 MHz, $CDCl_3$): 2.88 (3H, s), 3.71 (3H, s), 3.91 (6H, s), 6.72 (1H, s), 6.83–6.88 (2H, dd), 8.73 (1H, s). ^{13}C NMR (125 MHz, $CDCl_3$): 26.9, 34.1, 42.2, 52.4, 56.2, 56.5, 62.4, 62.8, 111.4, 111.7, 111.9, 119.7, 124.2, 126.4, 127.8, 127.9, 143.3, 144.5, 150.3, 152.9. Compared to literature [20], compound **1** from zone III was identified as isocorydine.

Compound **2**: positive ESI-MS, m/z 342 (M+H). ^{1}H NMR (500 MHz, $CDCl_3$): 2.90 (3H, s), 3.71 (3H, s), 3.90 (6H, s), 6.70 (1H, s), 6.90 (1H, d), 7.11 (1H, d), 8.79 (1H, s). ^{13}C NMR (125 MHz, $CDCl_3$): 25.2, 33.1, 40.5, 51.8, 56.3, 56.3, 61.7, 62.3, 111.3, 111.8, 119.3, 121.1, 121.9, 125.2, 125.7, 127.3, 143.8, 144.1, 150.8, 152.6. Compared to literature [20], compound **2** from zone IV was identified as corydine.

Compound **3**: positive ESI-MS, m/z 356 (M+H). ^{1}H NMR (500 MHz, $CDCl_3$): 2.90 (3H, s), 3.71 (3H, s), 3.90 (6H, s), 6.70 (1H, s), 6.90 (1H, d), 7.11 (1H, d), 8.79 (1H, s). ^{13}C NMR (125 MHz, $CDCl_3$): 25.2, 33.1, 40.5, 51.8, 56.3, 56.3, 61.7, 62.3, 111.3, 111.8, 119.3, 121.1, 121.9, 125.2, 125.7, 127.3, 143.8, 144.1, 150.8, 152.6. Compared to literature [21], compound **3** from zone V was identified as tetrahydropalmatine.

Compound **4**: positive ESI-MS, m/z 282 (M+H). ^{1}H NMR (500 MHz, $CDCl_3$): 3.60 (3H, s), 3.85 (3H, s), 6.60 (1H, s), 7.18–7.28 (3H, m), 8.33 (1H, d). ^{13}C NMR (125 MHz, $CDCl_3$): 25.7, 34.3, 41.6, 53.3, 56.2, 60.5, 111.6, 121.6, 126.0, 127.2, 128.1, 128.4, 128.5, 128.9, 131.6, 133.3, 146.4, 153.9. Compared to literature [22,23], compound **4** from zone II was identified as N-methylasimilobine.

Compound **5**: positive ESI-MS, m/z 266 (M+H). ^{1}H NMR (500 MHz, $CDCl_3$): 6.06 (1H, s), 6.21 (1H, s), 6.84 (1H, s), 7.32 (1H, d), 7.37–7.40 (2H, m), 8.02 (1H, d). ^{13}C NMR (125 MHz, $CDCl_3$): 25.6, 33.1, 41.0, 52.3, 102.0, 108.4, 115.9, 122.1, 125.4, 127.4, 128.3, 128.9, 129.2, 130.6, 133.2, 143.6, 148.4. Compared to literature [24], compound **5** from zone I was identified as anonaine.

4. Conclusion

In conclusion, five isoquinoline alkaloids were isolated from crude extract of *S. yunnanensis* by pH-zone-refining CCC for the first time. The present study demonstrates that pH-zone-refining CCC is an effective technique for the preparative separation of isoquinoline alkaloids from gram quantities of crude extract from *S. yunnanensis*. The present method may be applied to purification of various isoquinoline alkaloids from natural products.

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